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14. ABSTRACT Despite the fact that there have been significant developments in anti-cancer technology, ovarian cancer still remains one of the leading causes of gynecological cancer deaths in the United States. To contribute towards novel approaches with minimal toxicity in treatment of ovarian cancer we proposed the present work, where we are integrating the field of nanotechnology with ovarian cancer cell's unique property of overexpressing folic acid receptor alpha (FR- α) to specifically target ovarian cancer. A cerium oxide nanoparticle, called Nanoceria (NCe), that has the ability to act as an anti-oxidant in similar capacity as biological anti-oxidants was selected. NCe in our hands exhibited efficacy in inhibiting ovarian tumor growth in vivo. Taking advantage of the nanotechnology, NCe was conjugated with molecules of folic acid to specifically target ovarian tumors as they are known to over-express folate receptor. This would result in specific killing of ovarian cells by NCe while minimizing toxicity to normal tissue. Dr Seal's laboratory (UCF) was able to successfully conjugate NCe to folic acid to prepare folic acid tagged Nanoceria called FA-NCe. FA-NCe was tested in vitro and was observed to have higher anti-growth activity against ovarian cancer cells. This indicates that when given in animals, it maybe more potent in inhibiting ovarian tumor growth.					
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INTRODUCTION

Despite the fact that there have been significant developments in anti-cancer technology, such as chemotherapy, vaccines and hormone therapy, ovarian cancer still remains one of the leading cause of gynecological cancer deaths in the United States. The current standard therapy of surgical removal followed by chemotherapy results in overall tumor reduction with survival rate less than 50%. Chemotherapy regimens for patients with ovarian cancer include cisplatin and paclitaxel for which most of the patients develop resistance and as side effect have severe toxicity to normal cells. Clearly, other novel approaches with minimal toxicity are urgently needed which may specifically target ovarian cancer cells with minimal or no effects on normal cells.

To join in this effort we proposed the present work, where we planned to integrate the field of nanotechnology with ovarian cancer cell's unique property of over-expressing folic acid receptor alpha (FR- α) specifically target ovarian cancer.

Our experimental nanoparticle is **Nanoceria (N_{Ce})**, a cerium oxide nanoparticle. Nanotechnology-based tools and techniques are rapidly emerging in the fields of medical imaging and targeted drug delivery. Cerium oxide is a rare-earth oxide that is found in the lanthanide series of the periodic table. Nanocrystalline cerium oxide (nanoceria) exhibits a blue shift in the ultraviolet absorption spectrum, the shifting and broadening of Raman allowed modes and lattice expansion as compared to bulk cerium oxide indicating its unique properties. Nanoceria has emerged as a fascinating and lucrative material in biomedical science due to its unique ability to switch oxidation states between (III) and (IV) depending upon the environment. The ability to switch between mixed oxidation states of nanoceria is comparable to biological antioxidants. This imparts nanoceria with a very important biological property of radical scavenging which can be tuned based upon the retention of oxygen vacancies (defects) and concentration of Ce³⁺ species in nanoceria (1). N_{Ce} synthesized in Dr. Seal's laboratory has been tailored to retain mixed valence states (3+ and 4+) with the size in the range of 3-5nm. The reversibility of oxidation state is the key property in making N_{Ce} a potent antioxidant, thereby eliminating the need for repeated dosage (2). When generated intracellularly as a consequence of normal metabolism or pathological states, free radicals can strip electrons from cellular macromolecules and render them dysfunctional. Previous studies have demonstrated that cerium oxide nano-particles possess excellent antioxidant properties and act as potent, regenerative free radical scavengers in biological systems (1-3). These regenerative antioxidant properties are due, in part, to the valence structure of the cerium atom combined with inherent defects in the crystal lattice structure, which are magnified at the nano-scale.

Our proposal was based on our preliminary findings (presently in communication with Plos One), where we demonstrated that N_{Ce} by itself had the ability to abate ovarian tumor growth in an *in vivo* nude mouse model implanted with intraperitoneal A2780 human ovarian cancer cells. In the present work we had proposed to conjugate N_{Ce} to folic acid in order to specifically target ovarian cancer cells, which over-express FR- α . Folate receptor- α (FR- α) is a folate-binding protein overexpressed in ovarian and several other epithelial malignancies that has been used as a target for imaging and therapeutic strategies. Folate or folic acid, that binds to FR- α is an essential vitamin and critical metabolite needed for the biosynthesis of amino acids, DNA, RNA and methylation reactions. Folic acid is of importance especially for one-carbon transfer processes mediated by enzyme systems involved in DNA synthesis (4). Increased expression of FR- α has been described in various tumor tissues, including ovarian, endometrial and breast cancer (5). While the function of FR- α in cancer is not fully understood, since, folates are critical metabolites for nucleotide synthesis and methylation reactions, its overexpression might confer a tumor growth advantage by increasing folate availability to cancer cells (5). Over 90% of nonmucinous ovarian cancers over-express FR- α (6).

Several strategies have been employed to target the folate receptor including the use of anti -FR- α antibodies or folic acid (FA) conjugates.

We had hypothesize that targeted delivery of folic acid (FA) conjugated nanoceria (NCe) will specifically target the ovarian cancer cell and further modification of folic acid conjugated nanoceria to cisplatin will result in specific targeting and delivery of cisplatin resulting in exclusive elimination of ovarian tumor cells *in vivo*.

Following two aims were proposed:

Aim1: Investigate the therapeutic efficacy of folic acid conjugated NCe (FA-NCe) in a preclinical mouse model of ovarian cancer.

Aim2: Investigate the therapeutic efficacy of folic acid-Cisplatin conjugated NCe (FA-NCe-C) in a preclinical mouse model of ovarian cancer.

Due to the change of institute for the PI and time required to set a new lab from scratch, the work is currently behind the proposed SOW. In the present report we present the ability of Dr Seal's laboratory to successfully conjugate folic acid with NCe and some *in vitro* data demonstrating its efficacy against ovarian cancer cells.

We and will be working at out complete capacity to make up for the lost time.

BODY OF WORK

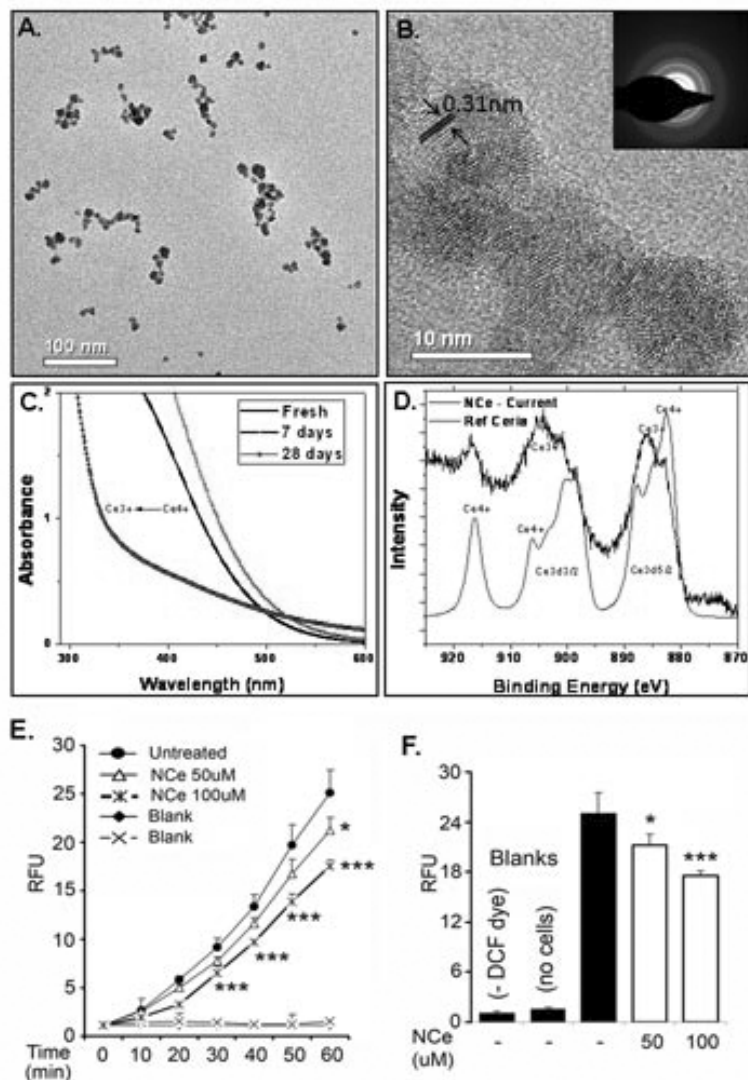
TIME LINE OF EVENTS:

The grant was awarded to the PI, Ramandeep Rattan, while at Mayo Clinic, Rochester MN 55905 in 2010. By the time all the paperwork involved was completed the PI had moved to Henry Ford Health System (HFHS), Detroit, MI 48202 in September of 2011. The grant was transferred to HFHS and all the paperwork and other conditions required had to be redone.

1. PI joined HFHS on September 1, 2011.
2. Grant was transferred to HFHS on November 1, 2011
3. The required IACUC protocol was approved from the HFHS IACUC committee on December 20, 2011 after which the ACURO form was submitted.
4. To gain Nanocerian from, Dr Seal at UCF, a subcontract was required as a prerequisite. This subcontract was completed by February 23, 2012
5. The ACURO was approved on MARCH 1, 2012
6. The first shipment of 500 ul of 5mM Nanoceria (NCe) and 100 ul of 5mM folic acid conjugated NCe (FA-NCe) arrived April 26, 2012
7. Ordered custom made folic acid free diet from BioServ end of May, 2012.

DATA:

1. Synthesis and characterization of cerium oxide nanoparticles: Cerium oxide nanoparticles used in this study contain individual crystallites of 3-5nm that are loosely agglomerated to 15-25nm. As the synthesis process is free from any organic surfactant the hard agglomeration of nanoparticles is controlled by tightly controlling the pH of the nanoparticles below 3.5 during synthesis to keep them in colloidal range. **Figure 1 A and B** shows the high resolution transmission electron micrographs (HRTEM) of NCe nanoparticles. It is evident from the image that nanoparticles are loosely agglomerated to about 15-25nm aggregates which could also be induced by the drying process. The hydrodynamic radius ($37.8 \text{ nm} \pm 0.8$) from the multimodal size distribution (volume %) analysis of DLS measurements agrees with the loose agglomerate size of the HRTEM analysis. High magnification image confirms the lattice planes of NCe in individual 3-5nm crystallites. UV-Visible spectroscopy was used to analyze the oxidation states of cerium oxide nanoparticles before and after aging treatment. **Figure 1C** shows the UV-Visible spectra from fresh and aged cerium oxide nanoparticles clearly indicating the predominance of Ce^{+3} oxidation state from the absorption peak at 252 nm as compared to absorption peak at 298 nm for Ce^{4+} . Further confirmation on the oxidation states of nanoparticles was obtained from XPS analysis (**Fig. 1D**). The XPS spectrum of cerium is very complex that contains multiple peaks from the spin orbit coupling of 3d orbitals (7). Several peaks in the Ce3d region that have been ascribed to $3d_{3/2}$ (899.5, 900.9, 903.5, 906.4 and 916.6) and



$3d_{5/2}$ (880.2, 882.1, 8885, 888.1 and 898) arising from multiple valence states of cerium. The spectrum from NCe shows a predominance of cerium in +3 oxidation state as depicted by the characteristic peaks at 880.2, 885.0, 899.5 and 903.5 eV. Taken together the data from characterization of NCe is consistent with previous reports wherein cerium can be retained in trivalent oxidation by decreasing the size of the nanoparticles (1-3, 8)

Figure 1: Preparation and characterization of NCe: High resolution transmission electron micrographs show the presence of **A** loose agglomerates of 15-20 nm at low magnification **B** individual 3-5nm crystallites. The d spacing of 0.31 nm shows the presence of planes of ceria while the selected area electron diffraction (SAED) pattern confirms the presence of fluorite lattice of cerium oxide. The trend in oxidation state of nanoceria in **C** shows that the synthesized nanoparticles have predominance of trivalent oxidation state that undergoes a slow transformation to Ce+3 oxidation state over a period of 28 days **D** x-ray photoelectron spectrum from a reference ceria sample in which cerium is predominantly in +4 oxidation state is compared to the spectrum from a 4 weeks aged sample of nanoceria demonstrating the high concentration of Ce in +3 oxidation states. **E**. Basal levels of ROS in ovarian cancer cell line.

A2780 cells were treated with NCe (50-100 μ M) for 48h. Cells were washed with PBS and loaded with DCF-DA dye (5 μ M) and fluorescence was recorded at excitation 485 nm and emission 530 nm for various time periods (5-60 min). Wells containing only cells without DCFDA dye (cross) or without cells containing DCFDA dye (filled diamond) were used as a blank. **F**. Bar graph represents ROS levels at the 60 min of treatment with DCF-DA dye. Results are shown as mean \pm S.D. of 4 samples. ***p< 0.001 NCe at 100 μ M; *p<0.05 NCe compared to untreated cells using two-tailed Student's t-test (Prism).

2. NCe treatment inhibits production of ROS levels in A2780 cell line: Cerium oxide

nanoparticles have been shown to act as free radical scavengers by inhibiting the production of reactive oxygen species (ROS) (7, 9,10). Since, it is well established that ROS accumulation plays an important role in initiation and progression of tumorigenesis in human ovarian cancer (11,12), we examined the effect of NCe on ROS generation in ovarian cancer cell line A2780. A2780 cell line was treated with NCe (50-100 μ M) and post 48h of treatment, ROS generation was measured using DCFH2-DA dye followed by fluorescence reading. As shown in **figure 1E, F**, NCe treatment significantly inhibited ROS levels in A2780 cell line, suggesting that NCe treatment inhibits basal levels of oxidative stress in A2780 OvCa cell line.

3. Synthesis and characterization of Ceria –Folic acid (FA-NCe):

Cerium oxide nanoparticles were synthesized by using precipitation method and as described above (13). Final nanoparticles were dried overnight under vacuum at 60-80°C. Cerium oxide nanoparticles (CNPs) obtained by this method were then functionalized with amine (CO₂-NH₂) by using 3-aminopropyl-trimethoxysilane as described elsewhere (14). Folic acid were conjugated to the amine functionalized by using 1-ethyl-3-[3-

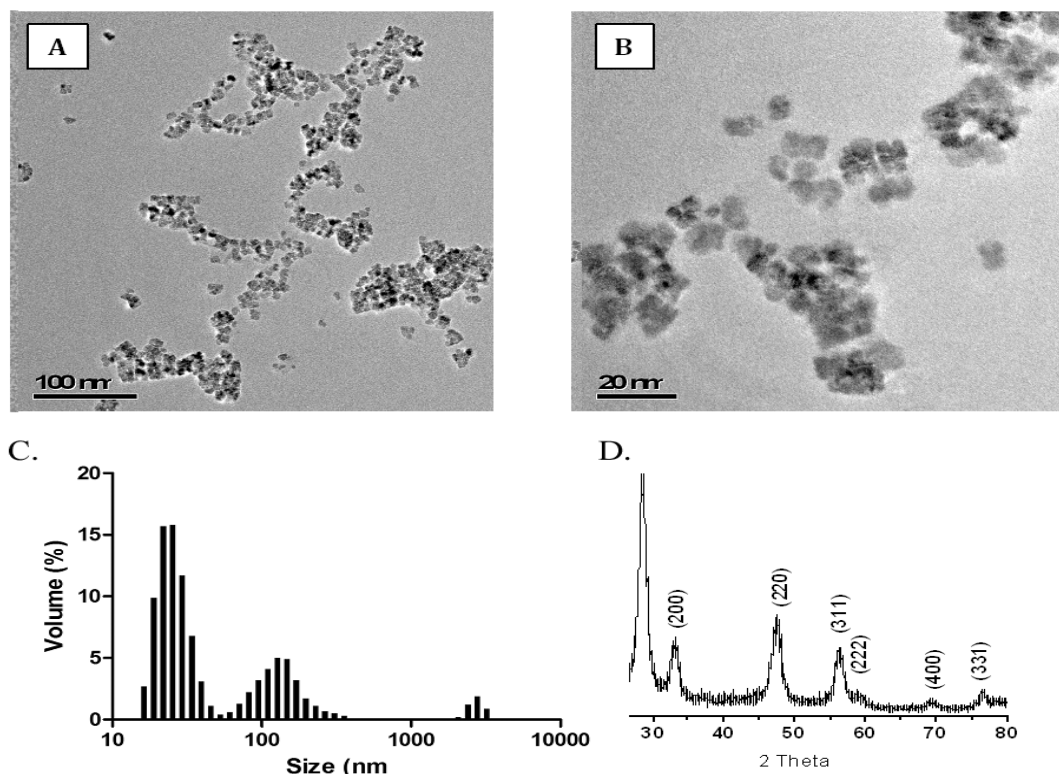


Figure 2: Characterization of FA-NCe. (A) and (B) are the HRTEM images of CNPs-FA conjugate, show particles size in the range of 8-10nm. (C) Hydrodynamic radii of the CNP-FA conjugates are shown. (D) XRD diffraction pattern of CNPs-FA conjugate.

dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) coupling chemistry. Molar ratio of folic acid:CeO₂-NH₂:Sulfo-NHS-EDC was 1:1:2.2:11.2. Reaction was carried for 24hr and then washed in both DMSO and water to remove EDC, Sulfo-NHS and unbound folic acid. Finally, folic acid conjugated CeO₂ (**FA-NCe**) were dried under vacuum at 40°C. CeO₂-FA was then characterized by using high resolution transmission electron microscopy (HRTEM), X-ray diffraction (XRD) and dynamic light scattering (DLS). HRTEM images show the size of the CeO₂-FA is ~10nm (**Fig. 2A, B**). Hydrodynamic radii of CeO₂ were found in approximately ~30nm nanometers with small percentage having 150nm agglomerated particles (Fig 2C). The surface charge of the CeO₂-FA has been also analyzed and found to be 25mV. XRD data confirm microcrystalline nature of the particles (Fig 2D).

4. FA-NCe inhibits ovarian cancer proliferation *in vitro*.

Our previous data with NCe had shown no effect on proliferation of ovarian cancer cells. Therefore we wanted to determine if FA-NCe has the ability to affect growth on ovarian cancer cells *in vitro*. For this cisplatin sensitive A2780 and resistant C200 were plated in 96 well plates at 4×10^3 cells/well and treated in triplicates with various concentrations of NCe (10-100 μ M). Cell viability was determined at 72hrs by MTT assay as described before (15). As shown in figure 3, NCe treatment had no significant effect on the proliferation or survival of ovarian cancer cell lines both A2780 (**Fig 3A**) or C200 (**Fig. 3B**). While FA-NCe showed significant inhibition in proliferation/cell death in both the cell lines (black bars).

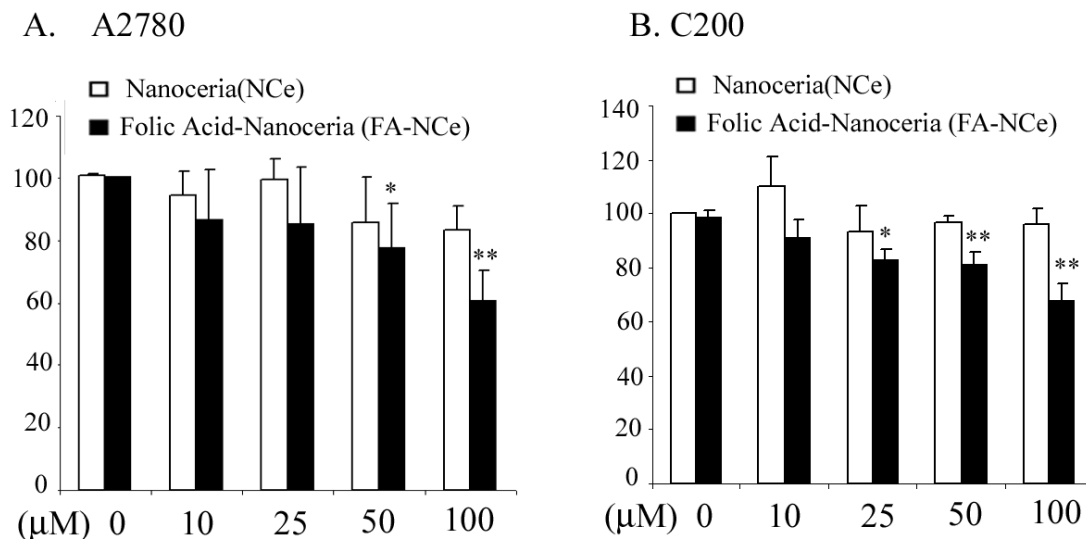


Figure 3: FA- NCe treatment inhibits proliferation in ovarian cancer cell lines.

Percent viability of **A.** A2780 and **B.** C200 cells treated with indicated doses of NCe (10-100 μ M) as determined by MTT assay. The data is represents three individual experiments done in triplicates. * $p < 0.001$ ** $p < 0.05$ compared to untreated (0) cells at respective time point using two-tailed Student's t-test (Prism).

5. FA-NCe show higher efficacy than NCe in inhibiting ovarian cancer colony forming ability.

To confirm our previous result we also performed the colony formation assay as it is considered a better assay to determine drug effect on cancer cells. For this, 2000 A2780 and C200 cells were plated in triplicates in 6-well plates and treated with indicated concentrations of NCe. The cells were allowed to form colonies for up to 2 weeks and media was replaced every fourth day. Colonies were stained with MTT and counted as described before (15). The clonogenic assay also showed similar results where NCe treatment showed no significant change, while FA-NCe significantly inhibited colony formation of both A2780 and C200 cell lines (**Fig. 4; black bars**).

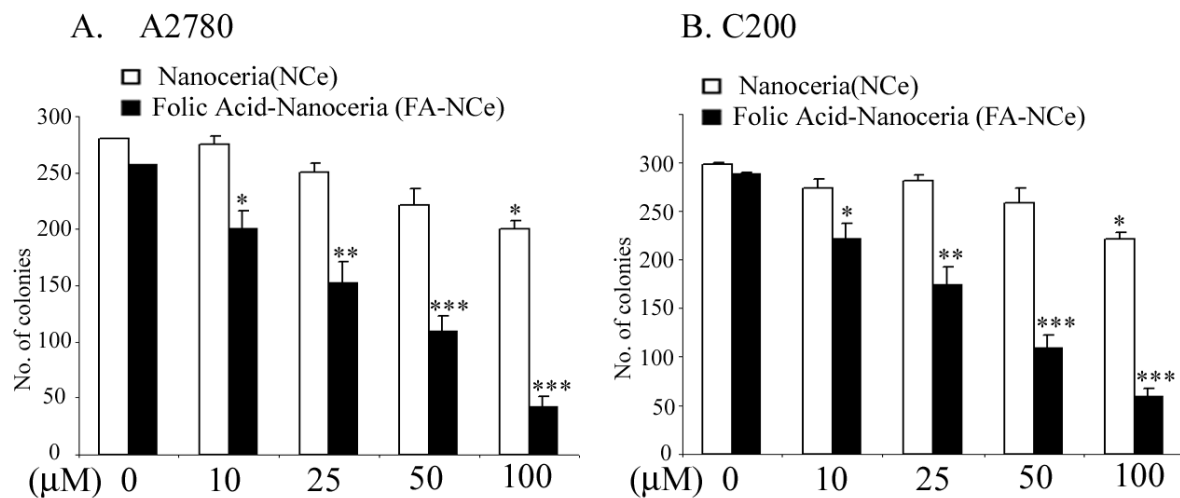


Figure 4: FA- NCE treatment inhibits colony formation in ovarian cancer cell lines.

Number of colonies formed in **A.** A2780 and **B.** C200 cells treated with indicated doses of NCE (10-100μM) as determined by visual counts after staining them with MTT dye. The data is represents three individual experiments done in triplicates. * $p < 0.001$ ** $p < 0.05$ compared to untreated (0) cells at respective time point using two-tailed Student's t-test (Prism).

Key Research Accomplishments:

1. Nanoceria with consistent anti-oxidant properties can be successfully synthesized.
2. Nanoceria can be successfully conjugated with folic acid.
3. Nanoceria conjugated with folic acid appears to have higher anti-tumor activity against cisplatin sensitive and resistant ovarian cancer cells *in vitro*.

REPORTABLE OUTCOMES:

1. Nanoceria can be successfully conjugated with folic acid
2. Nanoceria conjugated with folic acid appears to have higher anti-tumor activity against cisplatin sensitive and resistant ovarian cancer cells *in vitro*.

CONCLUSION

Nanoceria conjugated with folic acid appears to have higher anti-tumor activity against cisplatin sensitive and resistant ovarian cancer cells *in vitro*. This indicates that when given in animals, it maybe more potent in inhibiting ovarian tumor growth, which agrees with our hypothesis.

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